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Use of antagonists of the CB1 receptor for the manufacture of a composition useful for the treatment of hepatic diseases

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USE OF ANTAGONISTS OF THE CB1 RECEPTOR FOR THE MANUFACTURE OF A COMPOSITION USEFUL FOR THE TREATMENT OF HEPATIC DISEASES

FIELD OF THE INVENTION.

The invention relates to a new use for antagonists to the CB1 receptor for the manufacture of a composition useful for the treatment of hepatic diseases.

BACKGROUND OF THE INVENTION.

Liver fibrosis is the common response to chronic liver injury, ultimately leading to cirrhosis and its complications, portal hypertension, liver failure and hepatocellular carcinoma. The fibrogenic process is consecutive to intense proliferation and accumulation of hepatic myofibroblasts that synthesize fibrosis components and inhibitors of matrix degradation (Friedman, S.L., *J Biol Chem* 275, 2247-50 (2000)).

Cannabis Sativa contains over sixty compounds, the most active of which is (-) Δ^9-tetrahydrocannabinol (THC). Endogenous natural cannabinoids have also been characterized, anandamide and 2-arachidonyl glycerol, which are arachidonic acid-derived lipids (Piomelli, D *et al, Trends Pharmacol Sci* 21, 218-24. (2000)). Cannabinoids bind to two G protein-coupled receptors, CB1 and CB2, that equally bind THC (Pertwee, R.G., *Curr Med Chem* 6, 635-64. (1999)). CB1 is thus one of the two known cellular receptors for cannabinoids. This receptor being a G protein-coupled transmembrane receptor is known to be expressed in brain and blood vessels (Pertwee, R.G., *Curr Med Chem* 6, 635-64 (1999)) but not in hepatocytes (Guzman, M & Sanchez, C., *Life Sci* 65, 657-64 (1999)). CB1 mediates the psychoactive effects of cannabis. In contrast, CB2 receptors are mainly expressed in the immune system and are devoid of psychoactive effects (Friedman, S.L., *J Biol Chem* 275, 2247-50 (2000)). In addition to their psychotropic effects, cannabinoids display analgesic, antiemetic and orexigenic central effects (Harrold, J.A. & Williams, G. *Br J Nutr* 90, 729-34 (2003)). Moreover, cannabinoids also elicit anti-inflammatory and vasorelaxing properties (Kumar, R.N., Chambers, W.A. & Pertwee., *Anaesthesia* 56, 1059-68. (2001)). Several studies also suggest that cannabinoids may be potential antitumoral agents, owing to their ability to induce the regression of various types of experimental

tumors, including glioma or skin tumors. These antitumoral effects are mainly attributed to their antiproliferative and apoptotic properties (Bifulco, M. *et al.*, *Faseb J* 29, 29 (2001); Casanova, M.L. *et al.*, *J Clin Invest* 111, 43-50. (2003); Sanchez, C. *et al.*, *Cancer Res* 61, 5784-9. (2001)).

There are only few data concerning the hepatic action of cannabinoids. CB1 and CB2 receptors are not expressed in hepatocytes (Guzman, M. & Sanchez, C. *Life Sci* 65, 657-64 (1999)). However, CB1 receptors are present in endothelial cells isolated from hepatic arteries, and their expression increase during cirrhosis (Batkai, S. *et al. Nat Med* 7, 827-32. (2001)).

Two isoforms of the receptor CB1 have been isolated: a long isoform (corresponding to SEQ ID NO:1) and a shorter one truncated in the NH2 terminal part corresponding to a splice variant (corresponding to SEQ ID NO:2), which differ in their affinity for their ligands (Shire et al., *J Biol Chem*, (1995); Rinaldi-Carmona *et al*, J Pharmacol Exp Ther (1996)). There also exists 5 single nucleotide polymorphisms in the coding region of the CB1 receptor gene. Of these only three result in single amino acid changes to the CB1 receptor (these being, in SEQ ID NO:1, a Phenylalanine to Leucine substitution at position 200, an Isoleucine to Valine substitution at position 216 and a Valine to Alanine substitution at position 246 and the corresponding positions in SEQ ID NO:2). A consensus 7-domains sequence for the CB1 receptor exists which is strongly conserved in vertebrates but does not appear in other cannabinoid receptors (Attwood, T.K. and Findlay, J.B.C., *Protein Eng* 7(2) 195-203 (1994), Attwood, T.K. and Findlay, J.B.C., 7TM, *Volume 2 Eds G. Vriend and B. Bywater*, (1993), Birnbaumer, L., *Ann. Rev Pharmacol Toxicol*, 30, 675-705 (1990), Casey, P. J. and Gilman, A. G., *J. Biol.Chem.* 263(6) 2577-2580 (1988), 5. Attwood, T.K. and Findlay, J.B.C, *Protein Eng* 6(2) 167-176 (1993), Watson, S. and Arkinsall, S, *In The G Protein-Linked Receptor Factsbook, Academic press*, 1994, PP 80-83). That consensus amino acid sequence comprises the 7 protein domains of SEQ ID NO:3 to SEQ ID NO:9.

Antagonists to the receptor CB1, which include reverse or inverse agonists, have been previously described. These include the substituted amides described in WO03/077847, the substituted aryl amides described in WO03/087037, the substituted imidazoles described in WO03/063781, bicyclic amides described in WO03/086288, the terphenyl derivatives described in WO 03/084943, the N-piperidono-3-pyrazolecarboxamide and N-piperidino-5-(4-chlorophenyl)-1-(2, 4-dichlorophenyl)-4-methylpyrazole-3- carboxamide described in EP-B-656354, the aryl-

benzo[b]thiophene and benzo[b]furan compounds respectively described in US5596106 and US5747524, the azetidine derivatives described in FR2805817, 3-amino-azetidine described in FR2805810, or the 3-Substituted or 3,3-disubstituted 1-(di-((hetero)aryl)-methyl)-azetidine derivatives described in FR2805818. These documents are incorporated herein by reference. Other antagonists are commercially available such as *N*-(piperidin-1-yl)-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide, known commercially as AM251 and the compound known as LY-320135.

Uses of these CB1 receptor antagonists are known for the treatment of sexual dysfunction (patent application WO 03/082256), or diarrhoea (patent application WO 01/85092), or neuro-inflammatory diseases or substance abuse disorders, obesity, asthma, constipation (patent application WO 03/077847). Patent application WO 03/077847 discloses that CB1 antagonists can reverse the systemic hemodynamic alterations in rats with cirrhotic portal hypertension.

SUMMARY OF THE INVENTION.

There is no known involvement of either CB1 receptors or effects of CB1 antagonists in liver fibrogenesis originating from chronic liver diseases of any etiology (alcoholic, viral, toxic). Moreover, there is no known involvement of CB1 receptors or effects of CB1 antagonists in non-alcoholic steatohepatitis and liver carcinogenesis. However, the Applicant has discovered that CB1 antagonists have potent anti-fibrotic properties in the liver which can be used for the treatment of hepatic diseases. The invention thus relates to :

1. The use of an antagonist of the CB1 receptor in the manufacture of a composition for the treatment of hepatic diseases.

2. The use according to item 1 wherein the antagonist of the CB1 receptor is a specific antagonist of the CB1 receptor.

3. The use according to items 1 or 2 wherein the hepatic disease results in hepatic fibrosis.

4. The use according to items 1 to 3 wherein the hepatic disease is alcoholic liver cirrhosis, or chronic viral hepatitis or non-alcoholic steatohepatitis or primary liver cancer.

5. The use according to items 1 to 4 wherein the antagonist is N-piperidono-3-pyrazolecarboxamide or one of its pharmaceutically acceptable salt.

6. The use according to any of the preceding items wherein the CB1 receptor is selected from the group consisting of:

a) a protein having an amino acid sequence comprising SEQ ID NO:1 or a portion of SEQ ID NO:1, having the biological function of a G protein-coupled cellular receptor, capable of binding THC and transducing a cellular signal;

b) a protein having an amino acid sequence comprising SEQ ID NO:2 or a portion of SEQ ID NO:2, having the biological function of a G protein-coupled cellular receptor, capable of binding THC and transducing a cellular signal;

c) an allele of the protein having the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2, having the biological function of a G protein-coupled cellular receptor, capable of binding THC and transducing a cellular signal;

d) a protein having the amino acid sequence of SEQ ID NO:1 with a Phenylalanine to Leucine substitution at position 200; and/or an Isoleucine to Valine substitution at position 216; and/or a Valine to Alanine substitution at position 246;

e) a protein having the amino acid sequence of SEQ ID NO:2 with a Phenylalanine to Leucine substitution at position 139; and/or an Isoleucine to Valine substitution at position 155; and/or a Valine to Alanine substitution at position 185; and

f) a protein comprising the amino acid sequences of SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8 and SEQ ID NO:9 or amino acid sequences 80 % homologous to these, said protein having the biological function of a G protein-coupled cellular receptor, capable of binding THC and transducing a cellular signal.

7. The use according to items 1 to 5 wherein the CB1 receptor is a protein having a homology at the amino acid level with SEQ ID NO:1 of at least 45%, having the biological function of a G protein-coupled cellular receptor, capable of binding THC and transducing a cellular signal.

8. The use according to the preceding claim wherein the homology is at least 60%, preferably 70 %, more preferably 80 %, even more preferably 90 % and more preferably 95 %.

In a further embodiment the invention provides for :

9. The use of a nucleic acid sequence coding for a protein comprising SEQ ID NO:1 or SEQ ID NO:2 or a portion of SEQ ID NO:1 or a portion of SEQ ID NO:2, for the preparation of a composition for the treatment of hepatic diseases by the downregulation or suppression of the CB1 receptor.

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION.

The applicant has shown that the downregulation of CB1 receptors and the use of antagonist or reverse agonist to the CB1 receptor constitutes a treatment for various types of liver fibrosis. This is shown by several experiments.

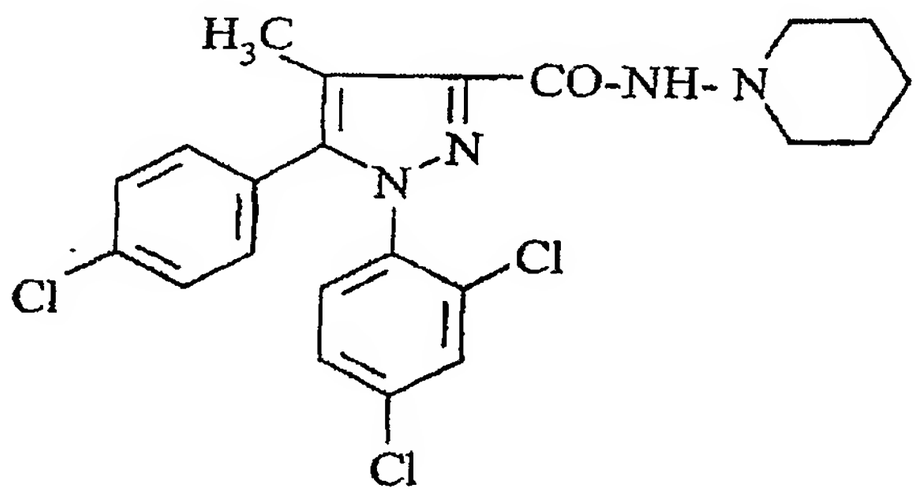
Firstly, the role of CB1 receptors in the progression of liver fibrosis was studied in CB1 receptor knock-out mice engineered to lack expression of CB1 receptors (CB1KO, n=15) and their wild type counterpart (WT, n=12) in a model of chronic carbon tetrachloride intoxication inducing fibrosis. Fibrosis and necroinflammation were assessed by a METAVIR-derived score. CB1KO mice showed reduced fibrosis compared to WT animals (fibrosis score: 2.59±0.13 vs 3.33±0.13, $p<0.05$). Accordingly, hepatic collagen, assessed by hydroxyproline determination was decreased by 40 % in CB1KO mice, as compared with WT animals (0.46±0.06 vs 0.73±0.11 mg/mg tissue, $p<0.05$). The necroinflammatory score was similar in both groups. Such inactivation of CB1 receptor is phenotypically equivalent to a perfect antagonistic block of the CB1 receptor by pharmaceutical means. Liver fibrosis is strongly reduced in CB1 knockout mice as compared to control mice, based on both histological analysis of the livers and measurement of hydroxyproline content, a specific biochemical marker of collagen deposition. Therefore, the inactivation of CB1 receptor reduces hepatic fibrosis.

Secondly, immunohistochemical labelling was performed with human normal, cirrhotic liver and cultured hepatic myofibroblasts. Immunochemistry showed a faint expression of CB1 receptors in normal liver (n = 3), contrasting with a marked upregulation in cirrhotic samples of various etiologies (n =13), predominating in nonparenchymal cells within and at the edge of fibrous septa. Double immunohistochemistry identified myofibroblasts as a main source of CB1 receptors, and accordingly, CB1 receptors were also expressed in cultured human hepatic myofibroblasts. CB1 receptors are faintly expressed by intrasinusoidal cells in the normal liver and are markedly upregulated during chronic liver diseases. Double immunohistochemistry revealed hepatic myofibroblasts as a prominent cell type expressing CB1 receptors in the cirrhotic liver. Therefore, CB1 receptor expression and activity correlate with hepatic fibrosis.

Thirdly, epidemiological studies were performed in a cohort of patients with chronic hepatitis C and showed that daily cannabis smoking is a risk factor for fibrosis progression in chronic hepatitis C as the rate of fibrosis is greater in these patients compared with non cannabis smokers. This demonstrates a participation of cannabinoid signal transduction in the fibrosis of the liver. Therefore the cannabinoid signalling pathway is involved in hepatic fibrosis.

Finally, in vitro studies of the effects of the CB1 receptor antagonist N-pipéridino-5-(4-chlorophényl)-1-(2, 4-dichlorophényl)-4-méthylpyrazole-3-carboxamide known commercially as SR141716 or rimonabant, were performed. This compound and its preparation are described in the European patent application EP656354-A1 and is represented by formula I:

(I)



Such an antagonist to CB1 and the pharmaceutically acceptable salts thereof can be prepared according to European patent application EP656354, and similarly the pharmaceutical compositions can be prepared according to the description of that same patent.

Suitable antagonists to the CB1 receptor can be specific to CB1 or not, and include reverse agonists. These antagonists also include the substituted amides described in WO03/077847, the substituted aryl amides described in WO03/087037, the substituted imidazoles described in WO03/063781, bicyclic amides described in WO03/086288, the terphenyl derivatives described in WO 03/084943, the N-piperidono-3-pyrazolecarboxamide and N-piperidino-5-(4-chlorophenyl)-1-(2, 4-dichlorophenyl)-4-methylpyrazole-3- carboxamide described in EP-B-656354, the aryl-benzo[b]thiophene and benzo[b]furan compounds respectively described in US5596106 and US5747524, the azetidine derivatives described in FR2805817, 3-amino-azetidine described in FR2805810, or the 3-Substituted or 3,3-disubstituted 1-

(di-((hetero)aryl)-methyl)-azetidine derivatives described in FR2805818, *N*-(piperidin-1-yl)-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (known as AM251) and the compound commercially known as LY-320135.

These results are not confined to humans and can be applied to mammals in general. In the pharmaceutical compositions of the present invention for oral, sublingual, subcutaneous, intramuscular, intravenous, transdermal, local or rectal administration, the active principle, alone or in combination with another active principle, can be administered in a unit administration form, as a mixture with conventional pharmaceutical supports, to animals and human beings. Suitable unit administration forms comprise oral-route forms such as tablets, gel capsules, powders, granules and oral suspensions or solutions, sublingual and buccal administration forms, aerosols, implants, subcutaneous, transdermal, intramuscular, intravenous and intranasal administration forms and rectal administration forms.

There exists various isoforms of the CB1 receptors and other variants. Accordingly the use of antagonists to any of these variant CB1 receptors would not take one outside of the field of the invention. Generally and in addition to protein sequence identity or homology, the biological function of a CB1 receptor can be defined by being a G protein-coupled cellular receptor, capable of binding THC and transducing a cellular signal. The skilled worker would be able to test the CB1 receptor function by known standard procedures. These could include the expression of putative CB1 cDNA in CHO cells, the subsequent testing using CB1 ligands and the measurement of the cell signalling activity of the activated putative CB1 receptor, for example by the measurement of cyclic AMP production.

An alternative to reducing the signal transduction of cannabinoids through the CB1 receptor by pharmacological means, involves the downregulation or suppression of that receptor. There exist various known techniques for downregulating or suppressing the expression of genes that the skilled worker will be able to use. A non-exhaustive list involves inactivation by homologous recombination (Gossen, J, trends Genet. 9:27-31, 1993), RNA interference (Elbashir S.M., Nature. 2001 May 24; 411(6836):428-9), the expression of dominant negative receptors (Dosil, M. *et al*, Mol Cell Biol. 1998 Oct, 18(10):5981-91) and the transgenic expression of suppressing transcription factors.

It is also possible to screen for CB1 antagonists with selective anti-fibrotic properties by using myofibroblast cultures obtained by outgrowth of explants prepared

from surgical specimens of normal human liver, as previously described (Li, L. *et al, Gastroenterology* 125, 460-9 (2003), Davaille J *et al., J Biol Chem* (2002)). In particular, a method of screening for CB1 antagonists with selective anti-fibrotic properties involves the steps of :

a) preparing multiple cell cultures of myofibroblasts,
b) exposing each of the cell cultures to one of the compounds to be tested,
c) assessing the effects of the compounds on the fibrogenic properties of the myofibroblasts, by evaluating their effects on the survival and growth of these cells,
d) selecting one the compounds tested.

An alternative method would be as above, wherein step c) involves assessing the effects of the compounds on the fibrogenic properties of the myofibroblasts, by evaluating their effects on their capacity to synthesize extracellular matrix and components that inhibit its degradation.

An alternative method would be as above wherein step c) involves assessing the effects of the compounds on the fibrogenic properties of the myofibroblasts, by evaluating cytokine production and migration.

The skilled worker would be able to perform these individual steps using well known procedures, such as described in Li, L. *et al, Gastroenterology* 125, 460-9 (2003), Davaille J *et al., J Biol Chem* (2002), Li L *et al., J Biol Chem* 2001 et Davaille, J. *et al., J Biol Chem* 275, 34628-33 (2000).

The compounds isolated by such means can then be used for the manufacture of a composition for the treatment of hepatic diseases.

EXEMPLES

Example 1: CB1 receptors upregulation in human cirrhotic liver

Materials.

Culture media and reagents were from Gibco (Invitrogen, France). Fetal calf serum was from JBio Laboratories (France). Pooled human AB positive serum was supplied by the National Transfusion Center. The rabbit anti-CB1 receptor antiserum (raised against residues 1 - 14 of the human CB1 receptor) and CB1 blocking peptide (residues 1 – 14 of the human CB1 receptor) were from Cayman (Spibio, France).

RNA Preparation and RT-PCR.

Total RNA was extracted from confluent quiescent cells in 100 mm dishes, using RNeasy kit (Qiagen, France). cDNA was synthesized from 2 µg of total RNA by reverse transcription for 1 h at 37°C, using 200 units of M-MLV reverse transcriptase (Invitrogen, France), in a 20 µl reaction mixture containing 0.05 µg/µl oligo $(dT)_{12-18}$ primers (Invitrogen, France), 0.5 mM dNTPs (Promega, France) and 10 mM dithiothreitol in first strand buffer (Invitrogen, France). To check for eventual genomic DNA contamination, controls were performed in the same conditions without reverse transcriptase. PCRs were performed with 2 µl of the reverse transcription reaction, using 1.25 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, France) and the corresponding buffer supplemented with 2 mM $MgCl_2$, 0.2 mM dNTPs, and 25 pmol of each primer in a total volume of 50 µl. 40 PCR cycles were carried out in a GeneAmp 2700 thermalcycler (Applied Biosystems, France), each cycle consisting of denaturation at 95°C for 45 s, annealing at 58°C for 45 s, and extension at 72°C for 30 s, with the first cycle containing an extended denaturation period (10 min) for the activation of the polymerase and the last cycle containing an extended elongation period (10 min). Oligonucleotide primers (MWG Biotech, France) for CB1 were as follows: CB1 sense primer 5'-TTTGGCTACACAATTGGAAGTCTAAGAACCC-3' and CB1 antisense primer, 5'-GCACACATTGACACGTATCCACTGCTTG-3', with a predicted PCR product of 287 bp. PCR amplified products were analyzed on a 1.5 % agarose gel, and blotted onto Hybond-N+ membrane (Amersham Pharmacia Biotech, France). After a prehybridization in a buffer containing 6XSSC, 5 mM EDTA pH 8, 5X denhardt, 0.1 % SDS and 0.1 mg/ml ssDNA, for 2 h at 42°C, the membrane was hybridized overnight at 42°C in the same buffer containing 50 ng of the CB1 oligonucleotide probe 5'-CCTGTGAGATGTGTATCAGTGTTTATGTGC-3', labeled with [γ-^{32}P] adenosine triphosphate, using T4 kinase (Invitrogen, France). After hybridization, the blot was washed twice in 0.1 % SDS, 1XSSC for 30 min at room temperature and analyzed by phospho-imager (Molecular Dynamics, France).

Human liver specimen.

Snap frozen surgical liver resections from 13 patients (8 men, 5 women, mean age 55 years, 39 - 72 range years) were retrospectively studied. Normal liver samples were collected from 3 women undergoing hepatic resection for colorectal metastasis (n = 3). Cirrhotic samples were obtained from 8 livers of patients undergoing liver transplantation and from 2 patients undergoing hepatic resection for hepatocellular carcinoma. Cirrhosis was consecutive to chronic HCV (n = 1) or HBV (n = 2) infections, primary biliary cirrhosis (n = 1), alcoholic liver disease (n = 4), or Wilson disease (n = 1) and remained cryptogenic in 1 case.

Immunohistochemical detection of CB1 receptors in normal and cirrhotic livers.

Frozen sections (5 – 7 μm) were air-dried and fixed in ice-cold acetone for 10 minutes at –20°C. Non specific binding was blocked by preincubating sections 1 h at room temperature with 20 % human serum in 50 mM Tris-buffered saline (TBS) pH 7.6. Sections were further incubated overnight at 4°C with a rabbit polyclonal antiserum to human CB1 receptor (Cayman, Spibio, France), diluted 1/2000 in antibody diluent (Dakopatts, France). After rinsing 3 times in TBS, sections were incubated for 45 min at room temperature with mouse monoclonal anti-rabbit immunoglobulin G antibodies (Dakopatts, France), diluted 1/50, rinsed 3 times in TBS, further incubated for 30 min at room temperature with rabbit anti-mouse immunoglobulin antibodies (Dakopatts, France), diluted 1/50, and then processed using the alkaline phosphatase-anti–alkaline phosphatase (APAAP) complex immunoenzymatic method, as described in the publication Li, L. *et al, Gastroenterology* 125, 460-9 (2003). To confirm the specificity of the primary antibody, controls included preadsorption of the primary antibody with the corresponding synthetic peptide (100 μg/ml, for 1 h at room temperature) or omission of the primary antibody. In order to determine whether hepatic myofibroblasts express CB1 protein, double immunostaining of CB1 and smooth muscle α-actin was performed. Sections were first processed for CB1 immunostaining using a standard three-stage biotin-streptavidin immunoperoxidase method. Briefly endogenous peroxidase was quenched by incubation of the acetone fixed sections in TBS/0.3 % H_2O_2 for 30 min, then washed in TBS. Non specific binding was blocked by preincubating sections 30 min with TBS/20 % human serum. Sections were then incubated for 15 min in avidin followed by 15 min in biotin (Vector Laboratories, Avidin/Biotin blocking kit), and further incubated over night at 4°C with the anti-CB1 antiserum. Subsequently, sections were washed in TBS and incubated successively with the secondary antibody biotinylated goat anti-rabbit (Dakopatts, France) (1/500) and streptavidin-horseradish peroxidase complex (1/50) (Pierce, Perbio, Interchim, France), 30 min each. Peroxidase activity was revealed using metal-enhanced diaminobenzidine (DAB) substrate (Pierce, Interchim, France). All steps were carried out at room temperature unless otherwise mentioned. Immunostaining for smooth muscle α-actin was then processed using the APAAP method described above, with a 1/5000 dilution of a monoclonal antibody to smooth muscle α-actin (Sigma, France). Slides were counterstained with aqueous haematoxylin. Single and double staining were visualized by bright-field photomicrographs on an Axioplan microscope (Zeiss, Oberkochen, Germany), equipped with a digital imaging system (Hamamatsu 3CCD color camera, Hamamatsu Photonics, France).

Isolation and culture of human hepatic myofibroblasts.

Human hepatic myofibroblasts were obtained by outgrowth of explants prepared from surgical specimens of normal liver obtained from surgery of benign or malignant liver tumors, as described in Davaille, J. *et al.*, *J Biol Chem* 275, 34628-33 (2000). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % serum (5 % fetal calf serum, and 5 % human serum, DMEM 5/5) and were used between the third and seventh passage. Experiments were performed on cells that were made quiescent by a 48 h incubation in serum-free Waymouth medium unless otherwise indicated. The myofibroblastic nature of these cells was evaluated as described in Davaille, J. *et al.*, *J Biol Chem* 275, 34628-33 (2000), and they display the phenotypic and functional characteristics of the fibrogenic cells found *in situ* during hepatic fibrogenesis (Win, K.M. *et al.*, *Hepatology* 18, 137-45 (1993). The cultures were found to express smooth muscle α-actin and two markers of hepatic myofibroblasts, fibulin-2 and interleukin-6 (Davaille, J. *et al.*, *J Biol Chem* 275, 34628-33 (2000)).

Immunocytochemical detection of CB1 receptors in cultured human hepatic myofibroblasts.

Human hepatic myofibroblasts were seeded (10,000/cm^2) in 35 mm dishes, grown in serum-containing medium for 24 h, serum-deprived for 48 h, washed with TBS and fixed in 4 % paraformaldehyde for 10 min. After washing once in TBS, cells were incubated in TBS containing 20 % human serum for 30 min at room temperature, and further incubated with the anti-CB1 antiserum (1/500 dilution in TBS/20 % human serum) for 3 h at room temperature and overnight at 4°C in a humid chamber. Cells were then rinsed extensively in TBS, incubated with a Cy3-conjugated goat anti-rabbit IgG (Sigma, France) (1/50 dilution in TBS/20 % human serum) at room temperature, in the dark, for 30 min, washed, covered with VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA), and viewed under fluorescence miscroscopy. To confirm the specificity of the primary antibody, controls included preadsorption with the corresponding synthetic peptide (100 µg/ml, for 1 h at room temperature) or omission of the primary antibody.

CB1 receptor expression was studied by immunohistochemistry with a polyclonal antibody directed against the human CB1 receptor, on frozen tissue sections prepared from surgical samples of normal (n =3) and cirrhotic livers (n =10), with

various etiologies (chronic HCV n = 1 or HBV n = 2, primary biliary cirrhosis n = 1, alcoolic liver disease n = 4 or Wilson disease n = 1 and remained cryptogenic in one case). In normal liver, a discrete, punctuate, CB1 immunoreactivity was detected along sinusoidal walls. In the cirrhotic liver, the overall intensity of the CB1 immunostaining markedly increased, irrespective of the etiology of cirrhosis. CB1 was mostly evident in numerous spindle-shaped cells distributed along the fibrotic septa. CB1 receptor expression was also found in other non parenchymal cells, including inflammatory cells and ductular proliferating cells located along the fibrotic septa. Specificity of the antibody was demonstrated by the lack of signal in sections incubated in the presence of the CB1 blocking peptide or in the absence of the first antibody.

Double immunohistochemistry, using an anti-CB1 receptor antibody and an anti-smooth muscle α-actin antibody, clearly identified hepatic myofibroblasts within fibrotic septa as a prominent cell type expressing CB1 receptors. Accordingly, CB1 receptors were also expressed in cultured human hepatic myofibroblasts, as demonstrated by both RT-PCR analysis and immunocytochemistry.

Example 2 : Reduced fibrogenic response in CB1 deficient mice

Animals and Experimental Design.

Male CD1 mice invalidated for CB1 receptors and wild type littermates were generated as previously described in the publication Ledent, C. *et al.*, *Science* 283, 401-4. (1999). Heterozygous mice were bred for more than fifteen generations on a CD1 background, before generating the wild type and mutant mice used in the present study. Forty male: WT (n = 20) and CB1-/- (n = 20), aged 8–10 weeks, were used and animals were divided into the following groups: WT sham (olive oil n = 8); WT CCl4 (n = 12); CB1-/- sham (olive oil n = 5); CB1-/- CCl4 (n = 15). Animals were housed in temperature and humidity controlled rooms, kept on a 12-h light/dark cycle and provided unrestricted amounts of food and water, unless otherwise specified. Fibrosis was induced by giving carbon tetrachloride (CCl4) (Sigma, St Quentin-Fallavier, France) mixed with olive oil (1 vol: 10 vol) at 0.5 ml/kg body weight, by intra peritoneal injection (IP), twice a week, in alterance with an equal volume of ethanol mixed with Cremophor and PBS (5/5/90) three times a week. Sham animals for CCl4 received olive oil. After 4 weeks, the animals were starved overnight and killed 48 h after the last CCl4 injection. Liver samples were taken from several lobes and either i) snap-frozen in liquid nitrogen and homogenized in RNA extraction solution, ii)

homogenized in H_2O and snap frozen in liquid nitrogen for hydroxyproline determination or iii) fixed in buffered formalin. Snap frozen sample were stored at –80°C until use. Blood samples were also collected in siliconed tubes containing inert gel barrier and clot activator disc (Venoject, Terumo, France), serum separated by centrifugation and stored at –20°C until use.

Liver-Function tests.

Routine liver-function blood tests (bilirubin, alkaline phosphatase, and aspartate transaminase) were performed on an automated analyzer.

Fibrosis, inflammation and necrosis assessment.

Liver specimen were fixed in 10 % formalin and paraffin-embedded. Tissue sections (4 µm-thick) were stained with hematoxylin-eosin (H&E) for routine examination, or with Picro-Sirius red for vizualization of hepatic collagen deposition. Histological grading (necrosis and inflammatory infiltration) and staging (fibrosis) were blindly assessed on at least 4 fragments from different areas of each liver, by an independent anatomopathologist. Fibrosis was staged on a scale of 0 to 4, according to a semi-quantitative modified METAVIR scoring system, as follows: no fibrosis = 0, portal fibrosis without septa = 1, few septa = 2, numerous septa without cirrhosis = 3, and cirrhosis = 4. Heterogeneity of fibrosis throughout the liver, when present, was taken into account by estimating the percent area corresponding to an individual score level in every fragment and by combining data for each liver. Necrosis, defined by acidophilic bodies, ballooning degeneration and/or scattered foci of hepatocellular necrosis, was graded as follows: absent= 0, mild = 1 (involvement of 1/3 of lobules or nodules), moderate (involvement of 1/3–2/3 of lobules or nodules) = 2, marked (involvement more than 2/3 of lobules or nodules) = 3. Inflammatory infiltration was graded from 0 to 3: none = 0, mild (portal and/or lobular) inflammatory infiltration in less than 1/3 of lobules or nodules = 1, moderate (portal and/or lobular) inflammatory infiltration involving 1/3-2/3 of lobules or nodules = 2, marked (portal and/or lobular) inflammatory infiltration involving more than 2/3 of lobules or nodules = 3. All samples were scored simultaneously.

Hydroxyproline content.

Hydroxyproline content was assessed as previously described in the publication Grenard, P *et al.*, *J Hepatol* 26, 1356-62. (1997). Three small fragments of each liver were pooled, homogenized in distilled water, lyophilized and overnight hydrolyzed in 6 N HCl at 110°C (10 mg of dry liver powder/ ml HCl 6N). The hydrolyzates were then treated with activated charcoal, filtered, evaporated and

resuspended in distilled water. Aliquots of the hydolyzates were used to measure hydroxyproline (HP) content spectrophotometrically by reacting with Ehrlich's reagent according to the method of Woessner (Woessner, J.F., *Arch Biochem Biophys* 93, 440-7 (1961)) modified as described in the publication Creemers, L.B *et al.*, *Biotechniques* 22, 656-8 (1997). The hepatic hydroxyproline content is expressed as micrograms per milligram of tissue (dry weight).

Statistics.

Results are expressed as mean ± SEM of n experiments. Results were analyzed by two-way analysis of variance (ANOVA) followed by paired comparisons corrected according to the Student-Newmann-Keuls method. $p < 0.05$ was taken as the minimum level of significance.

CB1 receptor-deficient mice (CB1-/-) and their wild type littermates (WT) were exposed to chronic CCl4 treatment. Sham mice (CB1-/- and WT) received olive oil. The mean body weight, liver weight and liver weight/body weight ratio did not significantly differ between the four experimental groups as shown in Table 1.

Table 1. Vital Parameters and Liver-Function Tests in mice wild type (WT) or CB1 deficient after 4 Weeks of CCl4 Treatment

Parameter	WT Olive oil	WT CCl4	CB1-/- Olive oil	CB1-/- CCl4
Body weight (g)	36.3±1.7	35.2±0.8	34.8±2.1	36.6±0.9
Liver weight (g)	1.9±0.1	2.1±0.1	1.8±0.1	2.1±0.1
Liver weight/ Body weight ratio(x100)	5.26±0.45	6.0±0.3	5.4±0.4	5.7±0.1
Aspartate transaminase (IU l-1)	94.7±9.2	1887.2±656.6 *	139.6±24.3	1341.9±457.1 #
Alkaline phosphatase (IU l-1)	37.9±5.9	55.0±6.2 *	33.6±2.0	56.4±4.6 #
Bilirubin Total	3.5±1.0	15.1±4.2 *	3.6±0.9	10.0±3.4 #

Results are expressed as mean ± SEM. * $P<0.05$ vs WT olive oil; # $P<0.05$ vs CB1-/- olive oil

WT mice developed liver fibrosis after 4 weeks of treatment with CCl4, as shown by histologic analysis of liver tissue sections stained with PicroSirius red. The livers of CCl4-treated WT mice showed numerous septa formation with some nodules. Strikingly, CCl4-treated CB1-/- mice showed weaker fibrogenic response with reduced formation of fibrotic septa. Accordingly, the fibrosis score was significantly lower in CCl4-treated CB1-/- mice as compared to the CCl4-treated WT group (2.59 ± 0.13 and 3.33 ± 0.13 respectively, $p<0.0\ 5$). Moreover, hepatic collagen content, assessed by liver hydroxyproline determination, was strongly reduced by 40 % in CCL4-treated CB1-deficient mice as compared to their WT counterparts (respectively 0.45 ± 0.06 and 0.73 ± 0.11 mg/mg of tissu (dry weight); $p<0.05$). Finally, necrosis and inflammation were not significantly different in CCl4-treated WT and CB1 -/- mice as shown in Table 2.

Table 2 : Necrosis and inflammation in wild type (WT) and CB1-/- mice after 4 Weeks of CCl4 treatment

Parameter	WT CCl4	CB1-/- CCl4
Necrosis	1.3±0.3	1.3±0.2
Inflammation	1.4±0.3	1.5±0.2

Results are expressed as mean ± SEM.

<u>Example 3 : Daily cannabis smoking is a risk factor for fibrosis progression in chronic hepatitis C</u>

195 consecutive naïve patients with dated exposure (men/women : 140/55, aged 42±10) were included. Data collected were consumptions of cannabis, alcohol and tobacco during disease duration, age at contamination, gender, route of transmission, genotype, BMI, steatosis, activity and fibrosis (METAVIR), and fibrosis progression rate (median value being 0.08 per year).

By univariate analysis fibrosis progression rate superior to 0.08/year was associated to cannabis smoking as shown in table 3, alcohol intake greater or equal than 30g/d (64%, $p = 0.03$), moderate or marked steatosis (65%, $p = 0.004$), age at contamination greater or equal than 25 (63 %, $p<0.01$), and histological activity greater or equal than A2 (65%, $p<0,001$). In multivariate analysis, fibrosis progression rate >0.08 was independently related to daily cannabis smoking (OR = 3.8; 95% CI (1.7-8.7)), alcohol intake greater or equal than 30 g/d (OR = 2.1; 95% CI (1.0-4.6)), age at contamination greater or equal than 25 (OR = 4.0; 95% CI (1.9-8.4)), and activity

greater or equal than A2 (OR = 7.5; 95% CI (3.5-16.1)). There is therefore a causal link between daily cannabis consumption and fibrosis progression.

Table 3: Incidence of Cannabis Consumption on Fibrosis progression

Cannabis	Fibrosis progression rate superior to 0.08/year	p
None, n = 102 (52%)	43 (42%)	
Occasional, n = 30 (16%)	15 (50%)	0.029
Daily, n = 63 (32%)	40 (63%)	

Example 4 : hepatic myofibroblast cultures in the presence of CB1 receptor antagonists

Apoptosis assays.

Apoptosis assays were performed on non-confluent cells allowed to attach overnight in DMEM 5/5 and serum-starved for 48 h, as described in Davaille J *et al.*, *J Biol Chem* 2002; 277:37323-30, and Li L *et al.*, *J Biol Chem* 2001; 276:38152-8. Nuclear morphology was assayed using DAPI staining, caspase-3- like activity was assayed on cell lysates using AC-DEVD-AFC as substrate, and DNA laddering was assayed by agarose gel electrophoresis of total DNA extracted using an Apoptotic DNA Ladder Kit.

Cell viability.

Cells (7, 000 cells/well in 96-well plates) were allowed to attach overnight in DMEM5/5, serum-starved for 48 h in DMEM without phenol red and treated with the indicated effectors for 16 h. CellTiter 96 AQueous One Solution reagent was added to each well and absorbance was recorded at 490 nm.

DNA synthesis.

DNA synthesis was measured in triplicate wells by incorporation of [3H] thymidine, as described in Tao J *et al.*, *J Biol Chem* 1999;274:23761-23769. Confluent quiescent cells were stimulated for 30 h with 20ng/ml PDGF-BB. [3H] thymidine (0.5 µCi/well) was added during the last 20 h of incubation.

The cell culture was performed as described in example 1 herein. Cell culture were exposed to increasing doses of CB1 receptor antagonist SR141716 and the proliferation, cell viability, DNA synthesis and apoptosis of hepatic myofibroblasts was measured.

CLAIMS

1. Use of an antagonist of the CB1 receptor in the manufacture of a composition for the treatment of hepatic diseases.

2. Use according to claim 1 wherein the antagonist of the CB1 receptor is a specific antagonist of the CB1 receptor.

3. Use according to claims 1 or 2 wherein the hepatic disease results in hepatic fibrosis.

4. Use according to claims 1 to 3 wherein the hepatic disease is alcoholic liver cirrhosis, or chronic viral hepatitis or non-alcoholic steatohepatitis or primary liver cancer.

5. Use according to claims 1 to 4 wherein the antagonist is N-piperidono-3-pyrazolecarboxamide or one of its pharmaceutically acceptable salt.

6. Use according to any of the preceding claims wherein the CB1 receptor is selected from the group consisting of:

   a) a protein having an amino acid sequence comprising SEQ ID NO:1 or a portion of SEQ ID NO:1, having the biological function of a G protein-coupled cellular receptor, capable of binding THC and transducing a cellular signal;

   b) a protein having an amino acid sequence comprising SEQ ID NO:2 or a portion of SEQ ID NO:2, having the biological function of a G protein-coupled cellular receptor, capable of binding THC and transducing a cellular signal;

   c) an allele of the protein having the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2, having the biological function of a G protein-coupled cellular receptor, capable of binding THC and transducing a cellular signal;

   d) a protein having the amino acid sequence of SEQ ID NO:1 with a Phenylalanine to Leucine substitution at position 200; and/or an Isoleucine to Valine substitution at position 216; and/or a Valine to Alanine substitution at position 246;

   e) a protein having the amino acid sequence of SEQ ID NO:2 with a Phenylalanine to Leucine substitution at position 139; and/or an Isoleucine to Valine substitution at position 155; and/or a Valine to Alanine substitution at position 185; and

   f) a protein comprising the amino acid sequences of SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8 and SEQ ID NO:9 or amino acid sequences 80 % homologous to these,

said protein having the biological function of a G protein-coupled cellular receptor, capable of binding THC and transducing a cellular signal.

7. Use according to claims 1 to 5 wherein the CB1 receptor is a protein having a homology at the amino acid level with SEQ ID NO:1 of at least 45%, having the biological function of a G protein-coupled cellular receptor, capable of binding THC and transducing a cellular signal.

8. Use according to the preceding claim wherein the homology is at least 60%, preferably 70 %, more preferably 80 %, even more preferably 90 % and more preferably 95 %.

9. Use of a nucleic acid sequence coding for a protein comprising SEQ ID NO:1 or SEQ ID NO:2 or a portion of SEQ ID NO:1 or a portion of SEQ ID NO:2, for the preparation of a composition for the treatment of hepatic diseases by the downregulation or suppression of the CB1 receptor.

ABSTRACT

The invention relates to the use of antagonists to the CB1 receptor for the preparation of a composition for the treatment of hepatic diseases and preferably to the use of N-piperidino-5-(4-chlorophenyl)-1-(2, 4-dichloropenyl)-4-methylpyrazole-3-carboxamide.

21978 Protein Sequences
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